

Amendments to the Specification:

Please amend the page number of the abstract to page "70."

Please replace the abstract on page 70 with the following abstract:

--The present invention relates to a chimeric expression promoter comprising at least one nucleic acid sequence derived from a first plant promoter containing a plant vascular expression promoter region, where the plant vascular expression promoter region is replaced with a nucleic acid sequence derived from a second plant promoter and containing a plant green tissue expression promoter region. The present invention also relates to a vector, a cell, and a transgenic plant containing the chimeric expression promoter, as well as a method of nucleic acid expression using the chimeric expression promoter.--

Please add the following paragraph at page 1, immediately following the title:

--This application is a Continuation of PCT/IB00/00370, filed March 29, 2000, which claims priority to FR 99/03925, filed March 29, 1999. The entire teachings of the above applications are incorporated herein by reference.--

Please replace the paragraph extending from lines 16-28 on page 34 with the following paragraph:

--In order to carry out the LCR reaction, 10 pmol of the phosphorylated oligodesoxynucleotides S1, S2, S5 and S7 were ligated in the presence of 10 pmol of the guide oligodesoxynucleotides G1, G2 and G4, 5 µl of Taq 10X DNA ligase buffer (New England Biolabs) and 40 units Taq DNA ligase (New England Biolabs). The ligation reaction was carried out in a thermocycle, sold under the tradename GeneAmp GENEAMP PCR System 9700 thermocycle (Perkin Elmer, Norwalk, USA). It consisted of a cycle of 1 min at 94°C, and 8 identical cycles each consisting of the succession of the following steps: 1 min at 65°C, 1 min at 57°C, 1 min at 52°C, 1 min at 48°C, 1 min at 43°C, and finally 10 min at 37°C. Then, the ligation reaction mixture was purified on a QIAquick column according to the supplier's recommendations.--

Please replace the paragraph extending from lines 23-31 on page 35 with the following paragraph:

--The 70 bp fragment containing the two "as-2" boxes and the "as-1" box were obtained by digesting 25 mg of plasmid pMRT1111 with 40 units of SpeI for 1 h at 37°C, then with 4 units of DraIII for 1 h at 37°C. The fragment was isolated by electrophoresis on a ~~Nu-Sieve~~ 3 % agarose gel sold under the tradename NU SIEVE (FMC, Rockland, USA) and finally purified on a QIAquick affinity column. The ends of this fragment were blunted by the action of Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the recommendations of the supplier, then purified again on a QIAquick affinity column.--

Please replace the paragraph extending from lines 17-21 on page 38 with the following paragraph:

--The 1.7 kb γ -zein promoter was obtained by digesting 15 μ g of plasmid p63 with restriction enzymes HindIII and BamHI for 1 h at 37°C. The thus liberated 1.7 kb γ -zein fragment was isolated on 0.8% agarose gel using a gel kit sold under the tradename ~~<<Concert~~ CONCERT Rapid Gel Extraction System ~~>>kit~~--

Please replace the paragraph extending from lines 28-36 on page 38 with the following paragraph:

--The ligation reaction was carried out with 50 ng of γ -zein promoter fragment and 100 ng of plasmid pMRT1126, in a reaction volume of 10 μ l, in the presence of tampon T4 10X DNA ligase and 400 units of T4 DNA ligase (New England Biolabs), in a thermocycle sold under the trade name ~~<<GeneAmp~~ GENEAMP PCR System 9700 ~~>>thermocycle~~ as described above. Previously prepared viable and competent *Escherichia coli* DH5 α bacteria were transformed with all of the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method--

Please replace the paragraph extending from lines 34 on page 39 to line 7 on page 40 with the following paragraph.

--In order to carry out the insertion, the pMRT1106 plasmid DNA (5 µg) was digested with AvrII enzyme, purified with the aid of a PCR purification kit sold under the tradename the <<QIAquick QIAQUICK PCR Purification>> kit, then dephosphorylated with 50 units of calf intestine alkaline phosphatase (New England Biolabs) in a final reaction mixture volume of 120 µl in the presence of 12 µl 3x 10 buffer (New England Biolabs) at 37 °C for 1 hour, isolated by electrophoresis on at 0.6% agarose gel in TBE buffer, purified with a gel extraction kit sold under the tradename <<QIAquick QIAQUICK Gel Extraction>> kit, dephosphorylated a second time with the calf intestine alkaline phosphatase under the conditions mentioned above, and finally purified with a PCR purification kit sold under the tradename <<QIAquick QIAQUICK PCR Purification>> kit and transferred to 50 µl de H₂O.--

Please replace the paragraph extending from line 8-15 on page 40 with the following paragraph:

--The PCR ligation reaction was carried out with 32,5 ng of digested dephosphorylated plasmid pMRT1106 and 50 ng of T-DNA fragments digested in a reaction mixture volume of 10 µl in the presence of 1 µl T4 10x DNA ligase buffer (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs). The ligation comprised 180 cycles each including 2 steps, the first one at 10°C for 30 seconds and the second step at 30°C for 30 seconds in a thermocycle sold under the trade name <<GeneAmp GENEAMP PCR System 9700>> thermocycle.--

Please replace the paragraph extending from line 21-31 on page 41 with the following paragraph:

--The expression cassette MPr1116 / *uidA*-IV2 / *nos* term was cloned at the modified HindIII site of the binary plasmid pGA492. It was obtained from the plasmid pMRT1116 digested with 80 units of PstI for 1 h at 37°C and purified on an affinity column sold under the tradename QIAQUICK-aQIAquick-affinity-column. The protruding 5' ends of this plasmid were blunted using Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the supplier's

recommendations. The plasmid thus modified was digested simultaneously with 80 units of EcoRI and 40 units of XmnI for 1 h at 37°C, then the 2.5 kb DNA fragment corresponding to the expression cassette was separated on 1% agarose gel and purified on an affinity column sold under the tradename QIAQUICK ~~a QIAquick affinity column~~.--

Please replace the paragraph extending from lines 15-30 on page 44 with the following paragraph:

--The ligation was carried out in a thermocycle sold under the trade name ~~(GeneAmp~~ GENEAMP PCR System 9700[[]]) by mixing 100 ng of binary plasmid pMRT1118 prepared as described above and 50 ng of expression cassette in a reaction volume of 12 µl in the presence of 1.2 µl T4 10X DNA ligase buffer (Epicentre Technologies), 1.2 µl of 25 mM ATP solution and 3 units of 10X DNA ligase (Epicentre Technologies). The ligation reaction consisted of a series of 200 identical cycles each consisting of a 30 sec step at 10°C and a 30 sec step at 30°C. Previously prepared, viable and competent *Escherichia coli* DH5α bacteria were transformed with half of the ligation reaction mixture. The plasmid DNA from the clones obtained, selected on LB media supplemented with kanamycin (50 mg/l), was extracted according to the alkaline lysis method and analysed by enzymatic digestion with the enzymes BamHI and EcoRI. The resulting clones were designated pMRT1185, pMRT1186, pMRT1187, and pMRT1188, and contain respectively the promoters MPr1162, MPr1164, MPr1165 and MPr1167.--

Please replace the paragraph extending from lines 9-31 on page 45 with the following paragraph:

--The ligation was carried out in the presence of 100 ng of binary plasmid, 50 ng of the PD35S CaMV fragment and 50 ng of the fragment corresponding to the *uidA*-IV2/term-nos sequence in a reaction volume of 20 µl, in the presence of T4 (1X) DNA ligase buffer and 400 units of T4 DNA ligase (New England Biolabs). The incubation was carried Out by PCR cycles in a thermocycle sold under the trade name ~~<<GeneAmp~~ GENEAMP PCR System 9700, ~~>>~~ ~~thermocycle~~ as described previously. Previously prepared, viable and competent *Escherichia coli* DH5α bacteria were transformed with half of the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with kanamycin (50 mg/l), was

extracted according to the alkaline lysis method and analysed by enzymatic digestion. The resulting plasmid was designated pMRT1182.--

Please replace the paragraph extending from lines 5-22 on page 46 with the following paragraph:

-- Ligation was carried out in a thermocycle sold under the trade name ~~(GeneAmp~~ GENEAMP PCR System 9700[[]]) by mixing 100 ng of binary vector pMRT1195 prepared as described above and 50 ng of expression cassette in a reaction volume of 12 µl in the presence of 1.2 µl of T4 10X DNA ligase buffer (Epicentre Technologies), 1.2 µl of 25 mM ATP solution and 3 units of 10X DNA ligase (Epicentre Technologies). The ligation reaction consisted in a series of 200 identical cycles each consisting of a 30 sec step at 10°C and a 30 sec step at 30°C. Previously prepared, viable and competent *Escherichia coli* DH5α bacteria were transformed with half of the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with kanamycin (50 mg/l), was extracted according to the alkaline lysis method and analysed by enzymatic digestion by the enzymes BamHI and EcoRI. The resulting clones were designated pMRT1245, pMRT1246, pMRT1247, pMRT1248, pMRT1249, pMRT1250, pMRT1251, pMRT1252 and pMRT1253, and contain respectively the promoters MPr1116, MPr1154, MPr1162, MPr1163, MPr1164, MPr1165, MPr1167, MPr1168 and MPr1169.--

Please replace the paragraph extending from lines 37-37 on page 46 with the following:

-- Ligation was carried out in a thermocycle sold under the trade name ~~(GeneAmp~~ GENEAMP PCR System 9700[[]])--

Please replace the paragraph extending from lines 32-37 on page 47 with the following paragraph:

--Ligation was carried out in a thermocycle sold under the trade name ~~(GeneAmp~~ GENEAMP PCR System 9700[[]]) by mixing 100 ng of binary vector pMRT1195 prepared as described above and 50 ng of expression cassette in a reaction volume of 12 µl in the presence of

1.2 µl of T4 10X DNA ligase buffer (Epicentre Technologies), 1.2 µl of 25 mM ATP solution and 3 units of 10X DNA ligase (Epicentre Technologies). The ligation--

Please replace the paragraph extending from lines 1-9 on page 49 with the following paragraph:

-- Twelve days after pollinisation (12 DAP), the corn seed was taken and sterilised in a 20% solution of bleach sold under the tradename bath of Domestos DOMESTOS ®, with agitation for 5 min. Following the elimination of the ~~Domestos~~ DOMESTOS ® by successive rinsing with deionized sterilised water, the pericarp and the aleurone cell layer were carefully removed under sterile conditions. Tangential cuts of the now exposed endosperm were made and placed on filter paper soaked in the minimal Murashige et Skoog media (MS 5524, Sigma).--

Please replace the paragraph extending from lines 15-23 on page 49 with the following paragraph:

--Twelve days after germination, the youngest leaves were taken and sterilised in a 20% solution of bleach sold under the tradename bath of Domestos DOMESTOS ®, with agitation for 5 min. The ~~Domestos~~ DOMESTOS ® was eliminated by successive rinsing with deionized sterilised water, then the leaves were placed for 24 h onto the weak plasmolysis media N6P6 0.4M (salts MS 3.98 g/l, vitamines N6 100 mg/l, L-proline 700 mg/l, casein hydrosylate 100 mg/l, saccharose 20 g/l, sorbitol 36,4 g/l, mannitol 36,4 g/l, 2,4 D 1 mg/l, pH 5.8, phytigel 3g/l), adlignous face up, in order to avoid splitting of the foliar cells during transformation.--

Please replace the paragraph extending from lines 25-30 on page 50 with the following paragraph:

-- The bombardment of tobacco leaves was carried out using a ~~Biolistic PDS-1000/He~~ gene gun system sold under the tradename BIOLISTIC PDS-1000/HE by following the general recommendations of the supplier (BioRad, Hercule, USA) relating to the manipulations and assembly of the various components of the apparatus. Each leaf was bombarded twice successively under the following shooting conditions :--

Please replace the paragraph extending from lines 29 to 36 on page 55 with the following:

--The bombardment of various corn tissues, and among others young leaves and albumen, was carried out with a ~~Biolistic PDS-1000/He~~ gene gun system sold under the tradename BIOLISTIC PDS-1000/HE using the general recommendations of the supplier (BioRad, Hercules, USA) in relation to the manipulations and assembly of the different components of the apparatus. Each endosperm was bombarded twice in succession with tungsten particles of 0.6 µm diameter, using the following shooting conditions:--

Please replace the two paragraphs extending from lines 21-29 on page 60 with the following:

--GUS activity was measured on 20 µl clarified crude leaf extract using a “GUS-Light chemiluminescent reporter gene assay” detection kit (Tropix Inc., Bedford, USA) according to the supplier’s recommendations. The measurement of light emission was carried out using a ~~Lumat~~ luminometer sold under the tradename LUMAT LB 9507 (EGG-Berthold, Bad Wildbad, Germany).

The quantity of total protein present in the crude extract was measured according to Bradford technique (1976), using a protein assay sold under the tradename “BioRad BIORAD protein assay” (BioRad, München, Germany).--

Please replace the Brief Description of the Figures on page 10, line 14 to page 12, line 23 with the following:

--The invention will be better understood through the following detailed description of one or more preferred embodiments, given purely as non-limiting examples, and with referral to the annexed drawing in which:

- Figures ~~I, II and III~~ 1, 2, and 3 schematically represent the structures of the comparative reference constructs, enabling a comparison of the chimeric promoters of the present invention with those already known and used. In figure ~~[[I]]~~ 1, the construction concerned contains the

reporter gene coding for [beta]-glucuronidase in the total absence of any promoter sequence as such, and thus useful as a negative control.

- Figure [[II]] 2 schematically represents a construct containing the [beta]-glucuronidase gene under the control of the CaMV double 35S promoter, useful as a strong reference control;

- Figure [[III]] 3 represents a construct useful as an internal reference for the transient expression experiments, and includes the reporter gene coding for a luciferase under the control of the CaMV 35S promoter;

- Figure [[IV]] 4 schematically represents the structure of several preferred embodiments of chimeric promoters produced in accordance with the present invention. The chimeric promoters MPr1116 and MPr1117 were obtained using the technique called lb-PCR. MPr1146 and MPr1147 were obtained by cloning the activator elements as1 and as2 from the CaMV promoter at the restriction enzyme site DraIII. The promoter MPr1154 was obtained through deletion of the two "as-1 like" sequences from the CoYMV promoter present in the 5' region of MPr1147. All of these promoters were cloned at the restriction sites PstI and BamHI into the vector pMRT1144 in order to obtain transcriptional fusion with the reporter gene uidA;

- Figure [[V]] 5 represents histochemical staining of tobacco leaves transformed with different promoters in accordance with the present invention. The tobacco leaves were transformed by a biolistic method using a gene gun sold under the tradename BIORAD PDS1000/HE ~~<<PDS1000/HE>> gene gun available from BIORAD~~, under the following conditions: split rupture disks at 900 psi, 2 µg of DNA bombarded over two successive firings, projectiles consisting of gold beads or spheres of about 1 µm in diameter, plant material positioned at 6 then 9 cm from the macrocarrier. After bombardment, the leaves were incubated in the dark in a culture chamber for 48 hours to enable expression of the reporter gene. The leaves were then incubated in a 0.1 M phosphate buffer containing 2 mg/ml of X-Glu at 37⁰ C. for 24 to 48 hours, then bleached in a 70% ethanol bath.

- Figure [[VI]] 6 represents a graph comparing the relative promoter activity of the different constructs after transient expression in tobacco leaves. Three days after bombardment

the leaves were ground then the crude extract clarified by centrifugation. The β -glucuronidase and luciferase activities were measured using fluorimetric methods on crude extract aliquots, then the ratio of GUS activity/LUC activity was determined. The histograms correspond to the average of the ratios for a given construct +/- standard mean error;

Figure [[VII]] 7 schematically represents other preferred embodiments of chimeric promoters according to the present invention, where:

the dark disk-shaped symbols represent the green tissue expression specific element;

the small white parallelepiped symbols represent the "endosperm like" boxes;

the small and large black hatched parallelepipeds represent respectively the "as-2" and "as-1" boxes from the CaMV promoter.

- Figure [[VIII]] 8 represents a comparison of the relative activity of the different promoters of the invention in transient expression experiments in corn albumen, where the β -glucuronidase and luciferase activities were measured by fluorimetry on an aliquot of crude extract. The histograms correspond to the average for a given construction +/- standard mean error;

- Figure [[IX]] 9 represents a comparison of the relative activity of the chimeric promoters MPr1116, MPr1146, MPr1167 and reference promoter MPr1092, evaluated in stable tobacco expression. Samples were taken from each primary transformant at 2, 4, 6, 8, and 10 weeks after transfer of the plants into the greenhouse. The β -glucuronidase activity was measured on each sample and weighted in relation to the total quantity of total protein. For each series of transformants, at a given time, the activities are classed in decreasing order and compared;

- Figure [[X]] 10 represents a comparison of the relative activity of the chimeric promoters MPr1162, MPr1164, MPr1165, MPr1167 and the reference promoter MPr1092, evaluated in stable tobacco expression. Samples were taken from each primary transformant at 2, 4, 6, 8, and 10 weeks after transfer to the greenhouse. The β -glucuronidase was measured on each sample and weighted in relation to the total quantity of protein. For each series of transformants, at a given times, the activities were classed in decreasing order and compared.--